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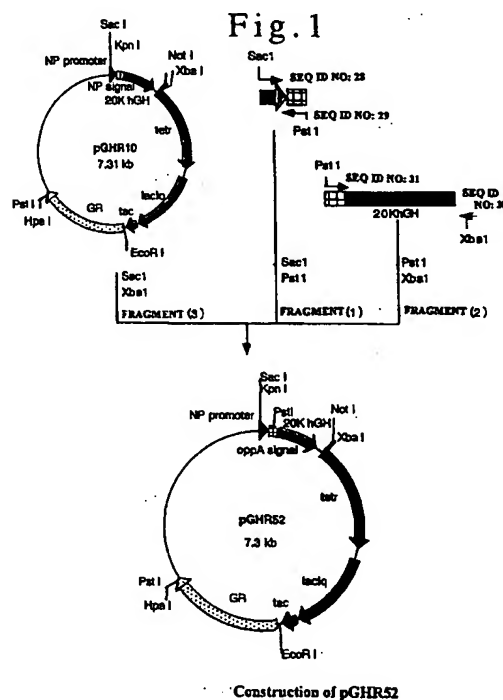
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(54) Method for secretory production of human growth hormone

(57) A DNA encoding 20K hGH is connected directly to a gene encoding Escherichia coli OppA protein secretion signal, or a modified form thereof, and a DNA encoding signal peptidase 1 to construct a recombinant plasmid. E. coli is transformed by said plasmid and cells of the resulting E. coli transformant strain are cultured for secretory production of the 20K hGH in the E. coli periplasm. This method enables efficient secretory production of 20K hGH and easy isolation and purification of 20K hGH from the periplasm fraction because the level of impure proteins in the E. coli periplasm is low.



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Description

[0001] The present invention relates to a method for secretory production of a human growth hormone having a molecular weight of about 20,000 using *Escherichia coli*. In particular, the present invention relates to a method for the production of growth hormone having a molecular weight of about 20,000, wherein *E. coli* is transformed by a recombinant plasmid carrying a DNA fragment in which the 5' end of a DNA fragment coding for a human growth hormone having a molecular weight of about 20,000 is linked in series to the 3' end of a DNA fragment coding for *E. coli* or *Salmonella* OppA secretion signal, or an amino acid modified form of *E. coli* OppA secretion signal, the resultant *E. coli* transformants are cultured, then the resultant culture fluid, cells or processed material thereof are used for the production.

[0002] Furthermore, the present invention relates to a method of producing a human growth hormone having a molecular weight of about 20,000, wherein *E. coli* is transformed with a recombinant plasmid carrying a DNA fragment coding for a human growth hormone having a molecular weight of about 20,000 and a DNA fragment coding for *E. coli* signal peptidase 1, the resulting *E. coli* transformants are cultured, then the resulting culture fluid, cells or processed materials thereof are used for the production.

[0003] Furthermore, the present invention relates to a method for increasing the production of a human growth hormone having a molecular weight of about 20,000 by altering the medium composition in culturing the transformants transformed by a recombinant plasmid with the abovementioned structure.

[0004] There are two known types of human growth hormone (hGH) derived from the pituitary gland: one having a molecular weight of about 22,000 (hereinafter referred to as 22K hGH) and the other having a molecular weight of about 20,000 (hereinafter referred to as 20K hGH).

[0005] 22K hGH is used for manufacturing pharmaceutical products, by means of recombinant DNA technology, for the treatment of pituitary dwarfism, pediatric chronic renal failure or the like. hGH has recently been found to have excellent activities such as immune promoting activity or lipolysis stimulating activity, as well as growth promoting activity. Broader applications are greatly expected in the future.

[0006] On the other hand, recently, the risks of side effects in the clinical use of 22K hGH are widely reported and have become important issues in securing the safety of GH in clinical use and in broadening its applications. Risks arising from the use of 22K hGH reported to date include possibilities of inducing leukemia and causing diabetes. There is a need for a growth hormone with a lower risk of side effects if its clinical applications are to be broadened.

[0007] 20K hGH has an amino acid sequence which corresponds to that of 22K hGH consisting of 191 amino acids except that 15 amino acid residues from the 32nd to the 46th inclusive from the N-terminal of 22K hGH are lacking. As compared to 22K hGH, this 20K hGH is reportedly low in leukemia cell proliferation activity and glucose intolerance, which is an index of diabetogenicity. Thus, the risks of side effects reported for 22K hGH should be less likely with 20K hGH. Also, according to results of in vitro experiments, 20K hGH has recently been found to be different from 22K hGH in its mode of binding to GH receptors. That is, M. Wada et al. revealed the difference between the two hGHs, showing that the binding activity of 22K hGH to a GH receptor decreases on the cell surface because 22K hGH binds to GH binding protein (GH receptor outer membrane protein) present in the serum at the level of physiological concentration, while a reduction in binding activity of 20K hGH to a GH receptor does not occur because 20K hGH hardly binds to a GH binding protein in physiological conditions, and that in a 1:2 complex (complex of 1 mole of growth hormone and 2 moles of GH receptor), which is necessary for the action of growth hormone via the GH receptor, 22K hGH forms a 1:1 complex (inactive type) while 20K hGH forms a 1:2 complex (active type) with the GH receptor (Mol. Endo 12, 1, 146-156, 1997). From these results, it is expected that 20K hGH may have higher activity than 22K hGH. It has also been revealed that as to lipolysis stimulating activity, which to date was not clear, 20K hGH is as active as 22K hGH (Japanese Patent Laid-open (Kokai) No. 97/216832, or European Patent Publication No. 0753307).

[0008] As mentioned above, 20K hGH has been found to be a growth hormone which exhibits lower risks and higher activity than 22K hGH and has become a novel growth hormone expected to be useful as a pharmaceutical product.

[0009] Recent developments in recombinant DNA technology makes it possible to produce heterologous proteins using microorganisms as host organisms. Generally, intracellular expression methods and secretory production methods are used for the production of proteins using microorganisms. In intracellular expression methods, proteins carrying methionine at the N-terminal are accumulated in the cytoplasm. In order to obtain proteins without the methionine residue at the N-terminal, it is necessary to enzymatically cleave amino acid sequences containing the methionine residue at the N-terminal, for example, by a peptidase. Further, proteins obtained by the intracellular expression methods are not active forms in their steric configurations and require refolding. Thus, intracellular expression methods are not necessarily recommended as efficient methods for protein production.

[0010] On the other hand, in secretory production methods using *E. coli*, target proteins are secreted and accumulated in the periplasm. Purification of proteins from the periplasm extract is easy because the level of impure proteins is lower than that in intracellular expression methods. Moreover, the secreted proteins have no methionine at the N-terminal and are naturally active forms in their steric configuration. Thus, secretory production methods are excellent.

[0011] However, secretory production methods using microorganisms require highly sophisticated techniques and thus rarely applied on an industrial scale. That is because precursor proteins synthesized in the cytoplasm pass through the cell membrane and because secretion signals have to be properly cleaved and removed by processing.

[0012] As to protein secretion in microorganisms, its mechanisms, relevant factors and their functions have been revealed. Based on these findings, various attempts have been made for efficient secretion methods.

[0013] Secretion signals are the first to be discussed. Secretion signals have an extremely important role in protein secretion to lead target proteins to the cytoplasmic membrane. It has been revealed that for the action of secretion signals, a positive charge in the positive charge region of their N terminals and hydrophobicity in the central hydrophobic area are essential (J. Biol. Chem., 267, 4882-4888, 1992). Based on this finding on structural characteristics of secretion signals, Udaka et al. made a modification in which a basic amino acid (Arg) was added to the N terminal positive charge region of a secretion signal of MWP (middle wall protein) derived from *Bacillus brevis* and a hydrophobic amino acid (Leu) was added to the central hydrophobic region in secretory production using *Bacillus brevis* as a host, and reported that the secretion efficiency of a sole fish growth hormone was improved to the level of 60 mg/L (Nippon Nogeikagaku Kaishi, 67(3), 372, 1993).

[0014] However, there are no theoretical guidelines for the modification of amino acid sequences of secretion signals and at present, preferable sequences have to be found by a try-and-error process. Further, there is no clear principle at present as to how to choose secretion signals derived from secretory proteins for target proteins for secretory production and thus, suitable secretion signals have to be found for the individual target proteins for secretory production.

[0015] The abovementioned problems are understood, for example, from the description in Japanese Patent Laid-open (kokai) No. 296491/94. It describes that the secretion signal derived from alkali phosphatase or enterotoxin, which are effective in secretory production of 22K hGH, is not necessarily effective in secretory production of several kinds of eukaryotic proteins. Namely, it reports that there were cases where no protein was synthesized, or proteins were expressed in the cytoplasm but the precursor proteins remained in the cytoplasm and not secreted depending on the combination of secretion signal and secretory protein.

[0016] Thus, it is necessary to select secretion signals appropriate to individual secretory proteins; however, to date, no secretion signal usable for secretion of any proteins has been found.

[0017] Secondly, the use of co-expression of proteins for the improvement of secretory production is discussed. To date, improvements in secretory production of proteins other than GH were reported, for example, for interleukin 6 by co-expression of SecE and prlA4 (a variant protein of SecY) (BIO/TECHNOLOGY, 12, 178-180, 1994) and human granulocyte-colony stimulating factor (G-CSF) by co-expression of a chaperon protein (Biochem. Biophys. Res. Commun., 210(2), 524-529, 1995).

[0018] As to secretory production of 20K hGH, a method in which glutathione reductase is co-expressed with 20K hGH (Japanese Patent Laid-open (Kokai) No. 322586/96; European Patent Publication No. 0735140), and a method in which *E. coli* Sec protein is co-expressed with 20K hGH (Japanese patent Laid-open (Kokai) No. 313191/97; European Patent Publication No. 0798380) were reported.

Summary of the Invention

[0019] The method for the production of 20K hGH by co-expression with glutathione reductase according to the abovementioned Japanese Patent (Kokai) Laid-open No. 322586/96 (or European Patent Publication No. 0735140) enabled the production of about 70 mg per 1 L of culture fluid. However, higher productivity is desired.

[0020] Although productivity of secretory production was thus improved by co-expression with glutathione reductase, studies by the present inventors revealed that in this method, a considerable amount of impure proteins other than the target protein, 20K hGH, is contained in the periplasm fluid extract obtained from the cells by an osmotic pressure shock method. As a result, purification of 20K hGH from the periplasm fluid extract comprises many steps, which will result in high production costs to purify 20K hGH to a medically acceptable grade.

[0021] An objective of the present invention is to provide a more efficient method for secretory production of 20K hGH using microorganisms, and another objective of the present invention is to provide a method for secretory production of 20K hGH using microorganisms, in which the level of impure proteins present in the periplasm fluid extract is decreased.

[0022] Objects of the present invention are to provide plasmids containing a DNA fragment in which the 5' end of DNA encoding 20K hGH links to the 3' end of DNA encoding a secretion signal which is a modified secretion signal of *E. coli* OppA, to provide *E. coli* transformants transformed by said plasmids, and to provide a method for secretory production of 20K hGH using said *E. coli* transformants.

[0023] Other objects of the present invention are to provide a recombinant plasmid having a DNA encoding 20K hGH and a DNA encoding *E. coli* signal peptidase 1, *E. coli* transformants transformed with said plasmid, and a method for secretory production of 20K hGH using said *E. coli* transformants.

[0024] To achieve the abovementioned objectives, the present inventors tried to modify a known strain described in

Japanese Patent Laid-open (Kokai) No. 322586/96 (or European Patent Publication No. 0735140), i.e., an *E. coli* transformant, MT-10765 (FERM BP-5020), which is capable of secretory production of 20K hGH using a modified secretion signal derived from a neutral protease of *Bacillus amyloliquefaciens* as a secretion signal and glutathione reductase derived from *E. coli* as a co-expression protein.

[0025] First, new secretion signals which could provide more efficient secretory production of 20K hGH were studied.

[0026] In order to confirm the effectiveness of new secretion signals, it is necessary to confirm their effect on the amount of secretory production of 20K hGH. The confirmation procedure comprises steps of constructing plasmids each containing a DNA fragment in which the 3' end of a DNA encoding a different secretion signal is connected to the 5' terminal of a DNA encoding 20K hGH, isolating *E. coli* cells transformed by said plasmids, culturing the resulting transformants, then measuring the secretory production of 20K hGH.

[0027] The present inventors attempted to increase productivity of 20K hGH by using secretion signals of proteins which are secreted in abundance in microorganisms. However, the inventors could not find secretion signals producing 20K hGH more efficiently by this method than by conventional methods.

[0028] Protein secretion is a physicochemical phenomenon in which proteins pass through the cell membrane in an energy-dependent manner. Therefore, the present inventors hypothesized that use of a secretion signal of a protein which is highly homologous to 20K hGH in its structure might be preferable. A protein having an amino acid composition highly homologous to 20K hGH and a protein having a behavior on a purification column similar to 20K hGH were selected as proteins homologous to 20K hGH in their structure. According to a homology search of amino acid sequences, TRBC (Meneewannakul, S. et al., *J. Bacteriol.*, 173, 3872-3878, 1991) and DPPA (Periplasmic Dipeptide Transport Protein Precursor (Dipeptide-binding Protein), Olson, E.R., et al., *J. Bacteriol.*, 173, 234-244, 1991) were selected as proteins highly homologous in their amino acid sequences. *E. coli* OppA (Periplasmic Oligopeptide Binding Protein, K. Kashiwagi et al., *J. Biol. Chem.*, 265, 8387-8391, 1990) which was revealed by the present inventors to be similar to 20K hGH in its behavior on ion exchange columns and affinity columns was selected as a protein similar to 20K hGH in its behavior on purification columns.

[0029] Studies on the effects of secretion signals of the abovementioned three kinds of proteins on secretory production of 20K hGH showed that the amounts of 20K hGH in secretory production with the secretion signals derived from TRBC and DPPA were less than 1 mg/L, namely there was no increase in secretory production of 20K hGH. Surprisingly, however, it was revealed that the amount of 20K hGH in secretory production using *E. coli* OppA secretion signal was more than that for MT-10765 (FERM BP-5020) described in Japanese Patent Laid-open (Kokai) No. 322586/96 (or European Patent Publication No. 0735140).

[0030] Furthermore, the present inventors tried to further improve secretory production of 20K hGH by modifying the secretion signal derived from OppA. Results showed that the use of a secretion signal in which an appropriate number of sequences of the basic amino acid, lysin, were inserted in the positively charged region of the N terminal sequence area and an appropriate number of sequences of the hydrophobic amino acid, leucine, were inserted in the central hydrophobic region attained a secretory production of 20K hGH of more than 110 mg per 1 L culture, namely a productivity higher than that with the use of the secretion signal derived from the natural type of OppA.

[0031] It is already known and not included within the scope of present invention that the *E. coli* OppA secretion signal has an effective role in protein secretion in secretory production in *E. coli*. However, it is impossible to predict whether the modification of OppA secretion signal would be extremely effective in secretory production of 20K hGH as mentioned above from previously known information.

[0032] The present inventors examined the improvement in co-expression of proteins as a secondary trial to achieve the objectives of the present invention.

[0033] As a result, the inventors found that the use of signal peptidase 1 derived from *E. coli* brought secretory production of 20K hGH to a level almost equal to that achieved with the use of glutathione reductase described in Japanese Patent Laid-open (Kokai) No. 322586/96 (or European Patent Publication No. 0735140).

[0034] Moreover, surprisingly, it was revealed that the rate of impure proteins other than 20K hGH present in the periplasm solution extracted from cultured cells by an osmotic pressure shock method was extremely low in co-expression with signal peptidase 1 as compared to that in co-expression with glutathione reductase. The reason was not clear but was speculated that signal peptidase 1 strongly suppressed the stress that cells receive by 20K hGH expression, which results in a decreased tendency for lysis of culture cells and thus a reduction in contamination of cytoplasmic proteins into the periplasm extract solution. This reduction of impure proteins in the periplasm solution facilitates the purification of 20K hGH from the periplasm fraction extract solution, and thus markedly contributes to an improvement in purification efficiency and lower costs in manufacturing 20K hGH.

[0035] Signal peptidase 1 is an enzyme which cleaves a secretion signal when a precursor protein produced in the cytoplasm passes through the membrane. Jan Maarten van Dijk et al. studied the effects of excessive co-expression of signal peptidase 1 on the secretory production of beta-lactamase when it was connected to various secretion signals (Jan Maarten van Dijk et al., *Mol. Gen. Genet.*, 227, 40-48, 1991). They reported that the effects of co-expression of signal peptidase 1 on the efficiency of processing (cleavage of signals) were different depending on the combination

of secretion signal sequences in the precursor and the target protein to be secreted

[0036] However, it is not at all obvious from the abovementioned report whether the co-expression of signal peptidase 1 is effective in improving the efficiency of secretion of 20K hGH, and moreover, it is not at all known that the tendency of cells to lyse is low such that the amount of impure proteins in the periplasm solution extracted from the cultured cells decreases in co-expression.

[0037] Furthermore, the present inventors altered culture conditions to determine a further improvement of secretory production of 20K hGH. As a result, the inventors succeeded in improving the production of 20K hGH up to as high as 82 mg/L by increasing the concentration of carbon source in the culture medium, using a known strain (MT-1 0765 (FERM BP-5020)) described in Japanese Patent Laid-open (Kokai) No. 32258/96 (also in European Patent Publication No. 0735140).

[0038] Accordingly, this method was further applied to MT-10852 (FERM BP-6290) and MT-10853 (FERM BP-6291) of the present invention. As a result, production was successfully increased by 4 to 5 times that of conventional production, i.e., to 350 mg/L using MT-10852 (FERM BP-6290) and to 350 mg/L using MT-10853 (FERM BP-6291), for example, with 3.5% glycerol and 1.5% succinic acid.

[0039] Further, the ratio of 20K hGH to total protein was compared, in which the ratio in a periplasm solution extracted from cells of a known strain, MT-10765 (FERM BP-5020), described in Japanese Patent Laid-open (Kokai) No. 32258/96 (or the specification of European Patent Publication No. 0735140) according to the method described in said specification is set to be 1. Results were 3.4 for MT-10853 (FERM BP-6291) in co-expression with glutathione reductase and 8.3 for MT-10852 (FERM BP-6290) in co-expression with signal peptidase 1. Thus, the productivity and purification process for 20K hGH extracted from MT-10852 (FERM BP-6290) and MT-10853 (FERM BP-6291) of the present invention was drastically improved as compared to that of the conventional method, which would enable 20K hGH production costs to be reduced.

[0040] The present invention was thus completed.

[0041] That is, the present invention comprises:

(1) a secretion signal which is more than 60% homologous to E. coli OppA secretion signal (excluding E. coli OppA secretion signal and Salmonella OppA secretion signal),

(2) a DNA encoding the secretion signal in (1) above,

(3) a recombinant plasmid containing a DNA fragment encoding a secretion signal having more than 60% homology to E. coli OppA secretion signal and a DNA fragment encoding a human growth hormone having a molecular weight of about 20,000, wherein the DNA encoding the human growth hormone having a molecular weight of about 20,000 is connected immediately after the DNA encoding said secretion signal,

(4) a transformant transformed by the recombinant plasmid of (3) above,

(5) a method of producing a human growth hormone having a molecular weight of about 20,000 characterized in that the transformant of (4) above is cultured, a periplasm fraction is extracted from the resulting cells, then the resulting periplasm fraction solution is purified to obtain a human growth hormone having a molecular weight of about 20,000,

(6) a recombinant plasmid containing a DNA fragment encoding a secretion signal, a DNA fragment encoding a human growth hormone having a molecular weight of about 20,000, and a DNA fragment encoding E. coli signal peptidase 1, wherein the 5' end of the DNA encoding the human growth hormone having a molecular weight of about 20,000 is connected to the 3' end of the DNA encoding said secretion signal,

(7) a transformant transformed by the recombinant plasmid of (6) above,

(8) a method of producing a human growth hormone having a molecular weight of about 20,000 characterized in that the transformant of (7) above is cultured, a periplasm fraction is extracted from the resulting cells, then the resulting periplasm fraction solution having a small amount of impure proteins is purified to obtain a human growth hormone having a molecular weight of about 20,000.

[0042] The present invention also includes a method of increasing production by altering the medium composition in the production of a human growth hormone having a molecular weight of about 20,000 by transformants transformed by the abovementioned recombinant plasmid, namely by using a medium containing at least one of 0.01-10% glycerol and 0.01-5% succinic acid.

[0043] The present invention provides a method for secretory production of 20K hGH using E. coli, which enables secretory production of 20K hGH in a larger amount than conventional methods.

Brief Description of the Drawings

[0044] Figure 1 shows the construction process for the 20K hGH secretion plasmid pGHR52.

[0045] Figure 2 shows the construction process for the 20K hGH secretion plasmids pGHR521-529.

[0046] Figure 3 shows the construction process for the 20K hGH secretion plasmid pGHS525.

[0047] Meanings of symbols in the drawings are as follows:

NP promoter: Promoter region of the neutral protease gene of *Bacillus amyloliquefaciens*

20K hGH: Human growth hormone having a molecular weight of about 20,000

GR: Glutathione reductase gene derived from *E. coli*

tac: tac promoter region

lacIq: lacI^q gene

tetr: Tetracycline resistance gene

oppA signal: Secretion signal of *E. coli* OppA protein

m-oppA signal: Modified secretion signal of *E. coli* OppA protein

SPI: Signal peptidase 1

rop: rop gene derived from pBR322

ori: Origin of replication derived from pBR322.

Detailed Description of the Invention and Preferred Embodiments

[0048] Secretion signals to be used in the present invention include secretion signals of OppA protein of *E. coli* or *Salmonella typhimurium* (*E. coli* OppA secretion signal or *Salmonella* OppA secretion signal) and modified secretion signals in which the amino acid sequence of *E. coli* OppA secretion signal is modified so as to maintain the function as a secretion signal and more than 60% homology (modified OppA secretion signals).

[0049] *E. coli* OppA secretion signal has the amino acid sequence of SEQ ID NO: 1 in the Sequencing Listing. Examples of modified OppA secretion signals include at least one of the following modifications:

- (1) insertion of 1 to 3 basic amino acids in the 2nd to 7th segment of SEQ ID NO: 1,
- (2) replacement of 1 to 3 acidic or neutral amino acids in the 2nd to 7th segment of SEQ ID NO: 1 with basic amino acids,
- (3) insertion of 1 to 9 hydrophobic amino acids at the 9th to 23rd of SEQ ID NO: 1, and
- (4) replacement of 1 to 9 non-hydrophobic amino acids in the 9th to 23rd segment of SEQ ID NO: 1 with hydrophobic amino acids.

[0050] Examples of the basic amino acid in the modifications in (1) and (2) include Lys and Arg and at least one of them can be used. As the hydrophobic amino acids in the modifications in (3) and (4), at least one amino acid selected from the group consisting of Leu, Ile, Val and Ala can be used.

[0051] Examples of the modified OppA signals include those having amino acid sequences of SEQ ID NOs: 2 to 27. Of these preferable modified OppA secretion signals of SEQ ID NOs: 2 to 27, the modified OppA secretion signal of SEQ ID NO: 23 is the most preferable example exhibiting the highest secretion productivity.

[0052] *Salmonella* OppA secretion signal has an extremely high homology to *E. coli* OppA secretion signal. *Salmonella* OppA secretion signal is already known and is not within the scope of the present invention. However, modified forms of *Salmonella* OppA secretion signal (for example, modified secretion signals having more than 60% homology in their amino acid sequence to *Salmonella* OppA secretion signal) are as effective as *E. coli* OppA secretion signal or modified forms thereof and thus modified *Salmonella* OppA secretion signals are within the scope of the present invention.

[0053] The recombinant DNA of the present invention has a structure in which the 5' end of a DNA encoding 20K hGH is directly connected to the 3' end of a DNA encoding *E. coli* or *Salmonella* OppA secretion signal or modified forms thereof, and can be expressed in a host to obtain a processor in which *E. coli* or *Salmonella* OppA secretion signal or a modified form thereof is connected to the N terminal of 20K hGH.

[0054] As the DNA encoding 20K hGH, any known DNA fragment including chemically synthesized DNA or cDNA based on the mRNA sequence of 20K hGH can be used. Furthermore, two different amino acids, serine and methionine, are reported for the 14th amino acid from the N-terminal amino acid sequence of 20K hGH. That is, Masuda, N. et al. (*Biophysica Acta*, 949, 125, 1988) reported that the cDNA base sequence coding for the 14th amino acid from the N terminal is AGT (a code for serine) while Martial, J.A. et al. (*Science*, 205, 602, 1979) reported that the RNA base sequence coding for the 14th amino acid from the N terminal of mRNA is AUG (a code for methionine). In the present invention, both DNAs coding for either amino acid can be used. Furthermore, 20K hGHs in which 1 to 5 amino acids in the amino acid sequence are replaced, deleted, lost or inserted within the range that the characteristics and functions of 20K hGH are not interfered with are also within the scope of the present invention.

[0055] The recombinant plasmid for the production of 20K hGH of the present invention can be constructed by incorporating this recombinant DNA into a plasmid such as pBR322 and pUC19 for *E. coli*, which has an origin of am-

plification amplifiable in the host cells in combination with a promoter which enables expression in the host cells. Any promoter can be selected, depending on the kind of host to be used, as long as it enables expression of the recombinant DNA in the host cells.

[0056] As the host, *E. coli* strains which are not pathogenic and are used ubiquitously are preferable, such as JM109, HB101 and W3110 (ATCC 27325). ATCC is an abbreviation for American Type Culture Collection.

[0057] An example of the recombinant plasmid has a promoter, a ribosome binding site, DNA encoding a secretion signal and DNA encoding 20K hGH, all connected in this order in the direction from the 5' end to the 3' end.

[0058] Signal peptidase 1 used for secretory production of 20K hGH by a co-expression system is one derived from *E. coli*. Further, the sequences in which one or two amino acids of the amino acid sequence of signal peptidase 1 derived from *E. coli* are replaced, inserted or deleted in the range that secretion of 20K hGH can be improved by co-expression as intended in the present invention are also within the scope of the present invention.

[0059] The signal peptidase 1 gene originally resides in the *E. coli* chromosome and thus the DNA encoding signal peptidase 1 derived from *E. coli* can be used. Accordingly, if *E. coli* is used as the host in the present invention, co-expression is attributed to the expression of the additional DNA which is artificially introduced besides that originating from the host.

[0060] Any promoter can be used as a promoter to express signal peptidase 1 as long as it functions in the host. Since the co-expression of signal peptidase 1 affects the level of secretion of 20K hGH, an excessive expression is not desirable. Therefore, it is preferable to use a method as that described in the Examples, which uses *lacR* to suppress the level of expression of signal peptidase 1 by the *tac* promoter. Furthermore, the co-expression of signal peptidase 1 can be carried out by incorporating a DNA encoding signal peptidase 1 and a DNA encoding 20K hGH into the same plasmid, incorporating a DNA encoding signal peptidase 1 and a DNA encoding 20K hGH into separate plasmids, or additionally incorporating a DNA encoding signal peptidase 1 into the *E. coli* chromosome DNA in which the signal peptidase gene naturally resides.

[0061] An example of the recombinant plasmid for co-expression constructed by incorporating a DNA encoding signal peptidase 1 and DNA encoding 20K hGH into the same plasmid has a structure in which a promoter, a ribosome binding site, a DNA encoding a secretion signal, a DNA encoding 20K hGH, a promoter and a DNA encoding signal peptidase 1 are connected in this order in the direction from the 5' end to the 3' end.

[0062] Any secretion signal which enables the secretion of 20K hGH can be used as a secretion signal to be incorporated into a recombinant plasmid for the co-expression system. Preferable examples include the abovementioned *E. coli* or *Salmonella* OppA secretion signal or modified forms thereof. The abovementioned DNA encoding 20K hGH and the promoter, the origin of replication and the like for the expression of DNA can also be used in this system.

[0063] The *E. coli* transformant of the present invention can be obtained by incorporating the abovementioned recombinant plasmid into a host by a known method. The host is transformed readily by the recombinant plasmid by an ordinary method, for example, by a method in which a competent cell is prepared by the rubidium chloride method, the resultant competent cell and the plasmid are mixed, then the mixture is subjected to heat shock at 42°C for 1 minute for incorporation of the plasmid into the host cell.

[0064] In the resulting transformant, a precursor in which the secretion signal and 20K hGH are connected is expressed under the control of the promoter and ribosome binding site sequence in the cytoplasm, the secretion signal is cleaved by signal peptidase 1 when this precursor passes through the inner membrane, then natural-type 20K hGH is secreted. When *E. coli* is used as the host, the natural-type 20K hGH is secreted and accumulated in the periplasm of *E. coli*.

[0065] Examples of the *E. coli* transformant of the present invention for secretory production of 20K hGH include MT-10853 (FERM BP-6291) and MT-10852 (FERM BP-6290). These strains are deposited with the abovementioned accession numbers according to the Budapest Treaty for International Agreement on Deposition of Microorganisms for Patent Procedure at the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba-shi, Ibaraki Prefecture).

[0066] *E. coli* transformants for secretory production of 20K hGH using *E. coli* as a host can be appropriately cultured in a flask or in a jar fermenter. The culture temperature is preferably 25-37°C, more preferably 28-30°C. Any medium used for an ordinary culture of *E. coli* can be used. A preferable culture medium is a 2-fold concentrated LB medium (20 g/L polypeptone, 10 g/L yeast extract) supplemented with 0.01-10%, preferably 0.1-5%, glycerol, and 0.01-5%, preferably 0.1-4%, more preferably 0.1-3%, succinic acid. The supplemented effect of glycerol and succinic acid is remarkable when added in combination. However, the effect was also found when either glycerol or succinic acid is added by itself.

[0067] The 20K hGH from *E. coli* transformants can be readily prepared by an ordinary method of extracting and purifying proteins from the periplasm. For example, an osmotic pressure shock method (Nossal G.N., *J. Biol. Chem.*, 241(13), 3055-3062, 1966) can be used. The extracted periplasm fraction can be purified by an ordinary method of purifying proteins, for example, a column chromatography method. In an ion exchange column chromatography method,

in which proteins first have to be loaded onto a carrier, the volume of the column can be advantageously small in the present invention because the periplasm liquid fraction contains a low level of impure proteins other than 20K hGH. [0068] The present invention will be illustrated in detail by the following examples; however, the invention is not intended to be limited to these examples.

Example 1: Study on usefulness of OppA secretion signal

[1] Construction of 20K hGH secretion plasmid containing OppA secretion signal gene

[0069] Oligonucleotides of SEQ ID NO: 28 and SEQ ID NO: 29 were made into double stranded chains by PCR (Polymerase Chain Reaction) and the resulting chains were cleaved with restriction endonucleases SacI and PstI to obtain a DNA fragment (1) encoding the promoter derived from *Bacillus amyloliquefaciens* neutral protease and the front-half of OppA secretion signal. Using secretion plasmid pGHR10 derived from strain MT-10765 (FERM BP-5020) described in Japanese Patent Laid-open (Kokai) No. 322586/96 as a template, a DNA fragment encoding the back-half of OppA secretion signal and 20K hGH was amplified by PCR using primers of SEQ ID NO: 30 and SEQ ID NO: 31. The resultant PCR product was cleaved with restriction enzymes PstI and XbaI to obtain a DNA fragment (2). Further, secretion plasmid pGHR10 was cleaved with restriction enzymes SacI and XbaI to obtain a vector-side fragment (3). These DNA fragments (1), (2) and (3), were ligated to construct secretion plasmid pGHR52. The process for these steps is shown in Figure 1.

[2] Construction of 20K hGH secretion plasmid containing modified OppA secretion signal gene

[0070] Using pGHR52 constructed in [1] as a template, a DNA fragment encoding the promoter derived from neutral protease of *Bacillus amyloliquefaciens* and a modified part of OppA secretion signal was amplified by PCR selectively using the oligonucleotide of SEQ ID No: 32 and various oligonucleotides of SEQ ID NOs: 33-39. The resulting PCR products were cleaved with restriction enzymes SacI and PstI to obtain fragments (4), (5), (6), (7), (8), (9) and (10). Next, the abovementioned fragments (2) and (3) and fragment (4) were mixed and ligated, then used for the transformation of *E. coli* cells (namely, fragment (4) is used in place of fragment (1) in Figure 1) to obtain secretion plasmid pGHR521. Similarly, secretion plasmid pGHR523 was obtained using fragment (5) in place of fragment (4), and secretion plasmids pGHR525, pGHR527, pGHR529, pGHR524 and pGHR526 were obtained using fragments (6), (7), (8), (9) and (10) in place of fragment (1), respectively (Figure 2).

[3] Evaluation of *E. coli* transformants transformed by 20K hGH secretion plasmid containing OppA secretion signal gene

[0071] Cells of *E. coli* W3110 strain (ATCC 27325) were transformed by secretion plasmid pGHR52 constructed in [1] to obtain an *E. coli* transformant strain, W3110/pGHR52.

[0072] Cells of this transformant strain were cultured on an LB agar medium supplemented with 10 µg/ml tetracycline at 30°C for 18 hours. The resultant cells were cultured in a liquid medium (20 g/L polypeptone, 10 g/L yeast extract, 10 g/L succinic acid, 10 g/L glycerol and 30 mg/L tetracycline) at 30°C for 24 hours. After culturing, a periplasm fraction containing secreted and accumulated 20K hGH was recovered from the cells by the osmotic shock method, and the 20K hGH concentration in the periplasm fluid fraction was measured by enzyme-immunoassay using an antibody against the human growth hormone. The amount of secreted 20K hGH per one liter of culture was calculated from the measurement. To compare the amount of secretory production, cells of MT-10765 (FERM BP-5020) were similarly cultured. Results are shown in Table 1.

Table 1:

Difference in the amount of 20K hGH in secretory production using different secretion signals		
Plasmid	pGHR10	pGHR52
Secretion signal	Derived from <i>Bacillus amyloliquefaciens</i> neutral protease (modified form)	Derived from <i>E. coli</i> OppA
Production (mg/L)	76	89

[0073] Results shown in Table 1 revealed that the *E. coli* transformant transformed by the plasmid carrying OppA secretion signal gene is superior to the *E. coli* transformant transformed by the plasmid carrying the gene for the modified secretion signal derived from *B. amyloliquefaciens* neutral protease in secretory production of 20K hGH.

[4] Evaluation of *E. coli* transformants transformed by plasmids carrying the genes for modified OppA secretion signals

[0074] Cells of *E. coli* W3110 strain (ATCC 27325) were transformed by secretion plasmids constructed in [2] to obtain *E. coli* transformant strains. Cells of resulting transformants were cultured as described in [3] and the amount of secretion was similarly measured. Results are shown in Table 2.

Table 2:

Difference in secretion of 20K hGH with various modified OppA secretion signals	
Plasmid	Production (mg/L)
pGHR521	89
pGHR523	100
pGHR524	115
pGHR525	120
pGHR526	108
pGHR527	90
pGHR529	89

[0075] Results shown in Table 2 revealed that the *E. coli* transformants transformed by the plasmids carrying the genes for modified OppA secretion signals were superior to the conventional *E. coli* transformant transformed by the plasmid carrying the genes for the modified secretion signals derived from *B. amyloliquefaciens* neutral protease in secretory production of 20K hGH. In particular, *E. coli* transformant MT-10853 (FERM BP-6291) transformed by pGHR525 carrying the gene for modified OppA secretion signal of SEQ ID NO: 3 showed the greatest secretory productivity.

Example 2: Study on usefulness of signal peptidase 1

[1] Construction of 20K hGH secretion plasmid for co-expression of signal peptidase 1 using modified OppA secretion signal

[0076] From the chromosomal gene of *E. coli* W3110, a DNA fragment encoding signal peptidase 1 was amplified by PCR using oligonucleotides of SEQ ID NO: 40 and SEQ ID NO: 41. The resultant PCR product was cleaved with restriction enzymes *MunI* and *PstI* to obtain a DNA fragment encoding signal peptidase 1 (11). This fragment (11) and a vector side fragment (12) which was created by cleaving pGHR10 described in Japanese Patent Laid-open (Kokai) No. 322586/96 with restriction enzymes *EcoRI* and *PstI* were mixed and ligated by an ordinary method, then used for transformation of *E. coli* W3110 (ATCC 27325) to obtain secretion plasmid pGHS15 (Figure 3). The resulting pGHS15 was cleaved with restriction enzymes *SacI* and *XbaI* to obtain a vector side fragment (13). Further, recombinant plasmid pGHR525 obtained in Example 1 was cleaved with restriction enzymes *SacI* and *XbaI* to obtain a 20K hGH expression cassette side fragment (14). Then, fragment (13) and fragment (14) were mixed and ligated to obtain secretion plasmid pGHS525 (Figure 3). This plasmid was used to transform *E. coli* W3110 strain to obtain transformant W3110/pGHS525 (MT-10852 (FERM BP-6290)).

[2] Evaluation of *E. coli* transformant transformed by plasmid carrying DNA for signal peptidase 1

[0077] Cells of transformant W3110/pGHS525 strain constructed in [1] were cultured in the same manner as described in Example 1 [3] and the amount of secreted 20K hGH was measured. The amounts of the total protein in periplasm fractions recovered from culture fluids of W3110/pGHR10, W3110/pGHR525 and W3110/pGHS525 by the method described in Example 1 [3] were measured by the method of Groves et al (Groves, W.E. et al., Anal. Biochem., 22, 195-210, 1968), and a comparison was made by setting the ratio of 20K hGH to the total protein in the periplasm fraction for W3110/pGHR10 as 1. Results are shown in Table 3.

Table 3:

Effect of use of signal peptidase 1 on secretory production of 20K hGH			
Plasmid	pGHR10	pGHR525	pGHS525
Production (mg/L)	76	110	115

Table 3: (continued)

Effect of use of signal peptidase 1 on secretory production of 20K hGH			
Plasmid	pGHR10	pGHR525	pGHS525
Ratio of 20K hGH to total protein in periplasm extract fluid fraction culture	1	1.6	4.8

[0078] Results shown in Table 3 revealed that the level of secretory production of 20K hGH by the E. coli transformants transformed with the recombinant plasmid, in which signal peptidase 1 was co-expressed, was higher than that by the transformants transformed with the conventional plasmid in which glutathione reductase was co-expressed, and the ratio of 20K hGH to the total protein in the periplasm extract fluid solution was greatly improved.

Example 3: Evaluation of 20K hGH productivity in altered culture conditions

[0079] Cells of E. coli transformants W3110/pGHR525 (MT-10853 (FERM BP-6291)) constructed in [4] of Example 1 and W3110/pGHS525 (MT-10852 (FERM BP-6290)) and the known strain W3110/pGHR10 (MT-10765 (FERM BP-5020)) described in Japanese Patent Laid-open (Kokai) No. 322586/96 (or European Patent Publication No. 0735140), for comparison, were cultured in an altered medium and the resultant cultures were evaluated.

[0080] Cells of each transformant strain were cultured on an LB agar medium supplemented with 10 µg/ml tetracycline at 30°C for 18 hours. The resultant cells were cultured in a liquid medium (20 g/L polypeptone, 10 g/L yeast extract, 15 g/L succinic acid, 36 g/L glycerol and 30 mg/L tetracycline) at 30°C for 42 hours. After culturing, the periplasm fraction containing secreted and accumulated 20K hGH was recovered from the cells by the osmotic pressure shock method, and the 20K hGH concentration in the periplasm fluid fraction was measured by enzyme-immunoassay using an antibody against the human growth hormone. The amount of secreted 20K hGH per one liter of culture fluid was calculated from the measurement. The amount of the total protein in each periplasm fraction was measured by the method of Groves et al. (Groves W.E. et al., Anal. Biochem., 22, 195-210, 1968). The ratio of 20K hGH to the total protein in the periplasm fraction for W3110/pGHR10, which was cultured and extracted for periplasm fraction by the method described in [3] of Example 1, was set to be 1. Results are shown in Table 4.

Table 4:

20K hGH production and ratio of 20K hGH to the total protein in the periplasm fluid extract in the altered medium composition				
Strain	W3110/ pGHR10	W3110/ pGHR10	W3110/ pGHR525	W3110/ pGHS525
Glycerol	10 g/L	36 g/L	36 g/L	36 g/L
Succinic acid	10 g/L	15 g/L	15 g/L	15 g/L
20K hGH production	76 mg/L	82 mg/L	350 mg/L	360 mg/L
Ratio of 20K hGH to the total protein in periplasm fraction	1	1.1	3.4	8.3

[0081] The present invention provides a method for secretory production of 20K hGH using E. coli cells, in which the level of secretory production of 20K hGH is higher than that attained by a conventional method.

[0082] Furthermore, the level of impure proteins in the periplasm extract solution obtained according to the present invention is low so that 20K hGH can be readily purified from the extract, therefore large amounts of highly pure 20K hGH can be produced at low cost.

[0083] Production efficiency of 20K hGH can be further improved by using a medium containing at least one of 0.01-10% glycerol or 0.01-5% succinic acid.

List of the sequences:

5 SEQ ID NO: 1

Met Thr Asn lle Thr Lys Arg Ser Leu Val Ala Ala Gly Val Leu
Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

10 SEQ ID NO: 2

Met Thr Asn lle Thr Lys Arg Ser Leu Leu Val Ala Ala Gly Val
Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

15 SEQ ID NO: 3

Met Thr Asn lle Thr Lys Arg Ser Leu Leu Leu Val Ala Ala Gly
20 Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

SEQ ID NO: 4

25 Met Thr Asn lle Thr Lys Arg Ser Leu Leu Leu Leu Val Ala Ala
Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

SEQ ID NO: 5

30 Met Thr Asn lle Thr Lys Arg Ser Leu Leu Leu Leu Leu Val Ala
Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

35 SEQ ID NO: 6

Met Thr Asn lle Thr Lys Arg Ser Leu Leu Leu Leu Leu Leu Val
Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu
40 Ala

SEQ ID NO: 7

45 Met Thr Asn lle Thr Lys Arg Ser Leu Leu Leu Leu Leu Leu Leu
Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala
Leu Ala

50 SEQ ID NO: 8

Met Thr Asn lle Thr Lys Arg Ser Leu Leu Leu Leu Leu Leu Leu
55 Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val

Ala Leu Ala

SEQ ID NO: 9

Met Thr Asn Ile Thr Lys Arg Ser Leu Leu Leu Leu Leu Leu
Leu Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn
Val Ala Leu Ala

SEQ ID NO: 10

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Val Ala Ala Gly Val
Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

SEQ ID NO: 11

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Val Ala Ala Gly
Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

SEQ ID NO: 12

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Leu Val Ala Ala
Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

SEQ ID NO: 13

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Leu Leu Val Ala
Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

SEQ ID NO: 14

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Leu Leu Leu Val
Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu
Ala

SEQ ID NO: 15

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Leu Leu Leu Leu
Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala
Leu Ala

SEQ ID NO: 16

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Leu Leu Leu Leu

Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val
Ala Leu Ala

5

SEQ ID NO: 17

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Leu Leu Leu Leu
Leu Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn
Val Ala Leu Ala

10

SEQ ID NO: 18

15

Met Thr Asn Ile Thr Lys Lys Arg Ser Leu Leu Leu Leu Leu Leu
Leu Leu Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly
Asn Val Ala Leu Ala

20

SEQ ID NO: 19

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Val Ala Ala Gly
Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

25

SEQ ID NO: 20

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Val Ala Ala
Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

30

SEQ ID NO: 21

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Leu Val Ala
Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu Ala

40

SEQ ID NO: 22

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Leu Leu Val
Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala Leu
Ala

45

SEQ ID NO: 23

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Leu Leu Leu
Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val Ala
Leu Ala

55

SEQ ID NO: 24

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Leu Leu Leu
Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn Val
Ala Leu Ala

SEQ ID NO: 25

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Leu Leu Leu
Leu Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly Asn
Val Ala Leu Ala

SEQ ID NO: 26

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Leu Leu Leu
Leu Leu Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala Gly
Asn Val Ala Leu Ala

SEQ ID NO: 27

Met Thr Asn Ile Thr Lys Lys Lys Arg Ser Leu Leu Leu Leu Leu
Leu Leu Leu Leu Val Ala Ala Gly Val Leu Ala Ala Leu Met Ala
Gly Asn Val Ala Leu Ala

SEQ ID NO: 28

ttcgagctcggtacccggaggtctagttttatattgcagaatgcgagattgctgggtttatt
atacaatataagttttcatt

SEQ ID NO: 29

cgctgcagctactaaacttctcttggtgatgttggtcatatgaaatccccctttttgaa
aataatgaaaacttatattgtat

SEQ ID NO: 30

aatttaactgtgat

SEQ ID NO: 31

gtagctgcaggcggttctggctgcgctaattggcaggggaatgtcgcgctggcattcccaact
ataccacttt cgcgcc

SEQ ID NO: 32

tgcaactttatccgc

SEQ ID NO: 33

gcctgcagctaccagacttcttttctttttggtgatgttggtcat

SEQ ID NO: 34

gcctgcagctaccaataacagacttcttttctttttggtgatgttggtcat

SEQ ID NO: 35

gcctgcagctaccaggagcaataacagacttcttttctttttggtgatgttggtcat

SEQ ID NO: 36

gcctgcagctacgagcagcaggagcaataacagacttcttttctttttggtgatgttggt
cat

SEQ ID NO: 37

gcctgcagctaccagcaggagcagcaggagcaataacagacttcttttctttttggtgat
gttggtcat

SEQ ID NO: 38

gcctgcagctacgagcaataacagacttcttttctttttggtgatgttggtcat

SEQ ID NO: 39

gcctgcagctaccagcaggagcaataacagacttcttttctttttggtgatgttggtcat

SEQ ID NO: 40

ttccaattgatggcgaatatgtttgccctg

SEQ ID NO: 41

tttctgcagttaatggatgccgccaatgcg

[0084] Each of the above DNA sequences begins with its 5' end.

Claims

1. A secretion signal which is different from *E. coli* or *Salmonella* OppA secretion signals but is more than 60% homologous to said *E. coli* OppA secretion signal in its amino acid sequence, characterized in that a human growth hormone having a molecular weight of about 20,000 can be secreted and produced using a microorganism having a recombinant DNA in which the 3' end of a DNA encoding said secretion signal is directly connected to the 5' end of a DNA encoding the human growth hormone having a molecular weight of about 20,000.
2. A secretion signal according to claim 1 having at least one of the following modifications of the sequence in the amino acid sequence of SEQ ID NO: 1 in the Sequencing Listing:

- (1) insertion of 1 to 3 basic amino acids in the 2nd to 7th segment of SEQ ID NO: 1,
 - (2) replacement of 1 to 3 acidic or neutral amino acids in the 2nd to 7th segment of SEQ ID NO: 1 with basic amino acids,
 - (3) insertion of 1 to 9 hydrophobic amino acids in the 9th to 23rd segment of SEQ ID NO: 1, and
 - (4) replacement of 1 to 9 non-hydrophobic amino acids in the 9th to 23rd segment of SEQ ID NO: 1 with hydrophobic amino acids.
3. A secretion signal according to claim 2 wherein the basic amino acids to be inserted or to be replaced in the 2nd to 7th segment of SEQ ID NO: 1 in the Sequence Listing are selected from Lys and Arg and the hydrophobic amino acids to be inserted or replaced in the 9th to 23rd segment of SEQ ID NO: 1 in the Sequence Listing are amino acids selected from the group consisting of Leu, Ile, Val and Ala.
 4. A secretion signal according to claim 3 having the amino acid sequence of any one of SEQ ID NOs: 2 to 27 in the Sequence Listing.
 5. A DNA encoding a secretion signal of any one of claims 1 to 4.
 6. A recombinant DNA containing a DNA encoding a secretion signal which is more than 60% homologous to said E. coli OppA secretion signal in the amino acid sequence and a DNA encoding a human growth hormone having a molecular weight of about 20,000, characterized in that the 5' end of a DNA encoding said human growth hormone having a molecular weight of about 20,000 and the 3' end of a DNA encoding said secretion signal are directly connected.
 7. A recombinant DNA according to claim 6 wherein the secretion signal having a DNA encoding a secretion signal which is more than 60% homologous to said E. coli OppA secretion signal in the amino acid sequence is a modified secretion signal of E. coli or Salmonella OppA secretion signal.
 8. A recombinant DNA according to claim 6 wherein the secretion signal having a DNA encoding a secretion signal which is more than 60% homologous to said E. coli OppA secretion signal in the amino acid sequence is the secretion signal of claim 2, 3 or 4.
 9. A recombinant plasmid characterized in that it contains a promoter and the recombinant DNA of claim 6, 7 or 8 and that said DNA is incorporated in a position such that expression is possible by said promoter.
 10. A recombinant plasmid according to claim 9 wherein said promoter enables the expression of said recombinant DNA in E. coli and further has the origin of replication in E. coli.
 11. An E. coli transformant which is transformed by the recombinant plasmid of claim 9 or 10.
 12. An E. coli transformant according to claim 11 wherein the E. coli transformant is FERM BP-6219.
 13. A method for producing a human growth hormone having a molecular weight of about 20,000, characterized in that cells of an E. coli transformant strain of claim 11 or 12 are cultured and a periplasm extract solution obtained by extracting a periplasm fraction from the resulting cells is purified to obtain a human growth hormone having a molecular weight of about 20,000, and optionally a medium containing at least one of 0.01-10% glycerol and 0.01-5% succinic acid is used in culturing cells of the E. coli transformant strain.
 14. A recombinant plasmid comprising a recombinant DNA in which the 5' end of a DNA encoding a human growth hormone having a molecular weight of about 20,000 is directly connected to the 3' end of a DNA encoding a secretion signal and a DNA encoding E. coli signal peptidase 1.
 15. A recombinant plasmid according to claim 13 wherein the DNA encoding a secretion signal is either a DNA encoding E. coli, or Salmonella OppA secretion signal, or a DNA of claim 5.
 16. A recombinant plasmid according to claim 14 or 15 further comprising a promoter which enables the expression of said recombinant DNA in E. coli, a promoter which enables the expression of a DNA encoding said E. coli signal peptidase 1 and an origin of replication in E. coli.

17. An *E. coli* transformant transformed by the recombinant plasmid of claim 15 or 16.

18. An *E. coli* transformant according to claim 17 wherein the *E. coli* transformant is FERM BP-6290.

5 19. A method of producing a human growth hormone having a molecular weight of about 20,000 characterized in that
cells of an *E. coli* transformant strain of claim 17 or 18 are cultured and a periplasm extract solution, which is low
in the impure protein level, obtained by extracting a periplasm fraction from the resulting cells is purified to obtain
a human growth hormone having a molecular weight of about 20,000, and optionally at least one of 0.01-10%
10 glycerol or 0.01-5% succinic acid is used in the medium for culturing cells of an *E. coli* transformant strain.

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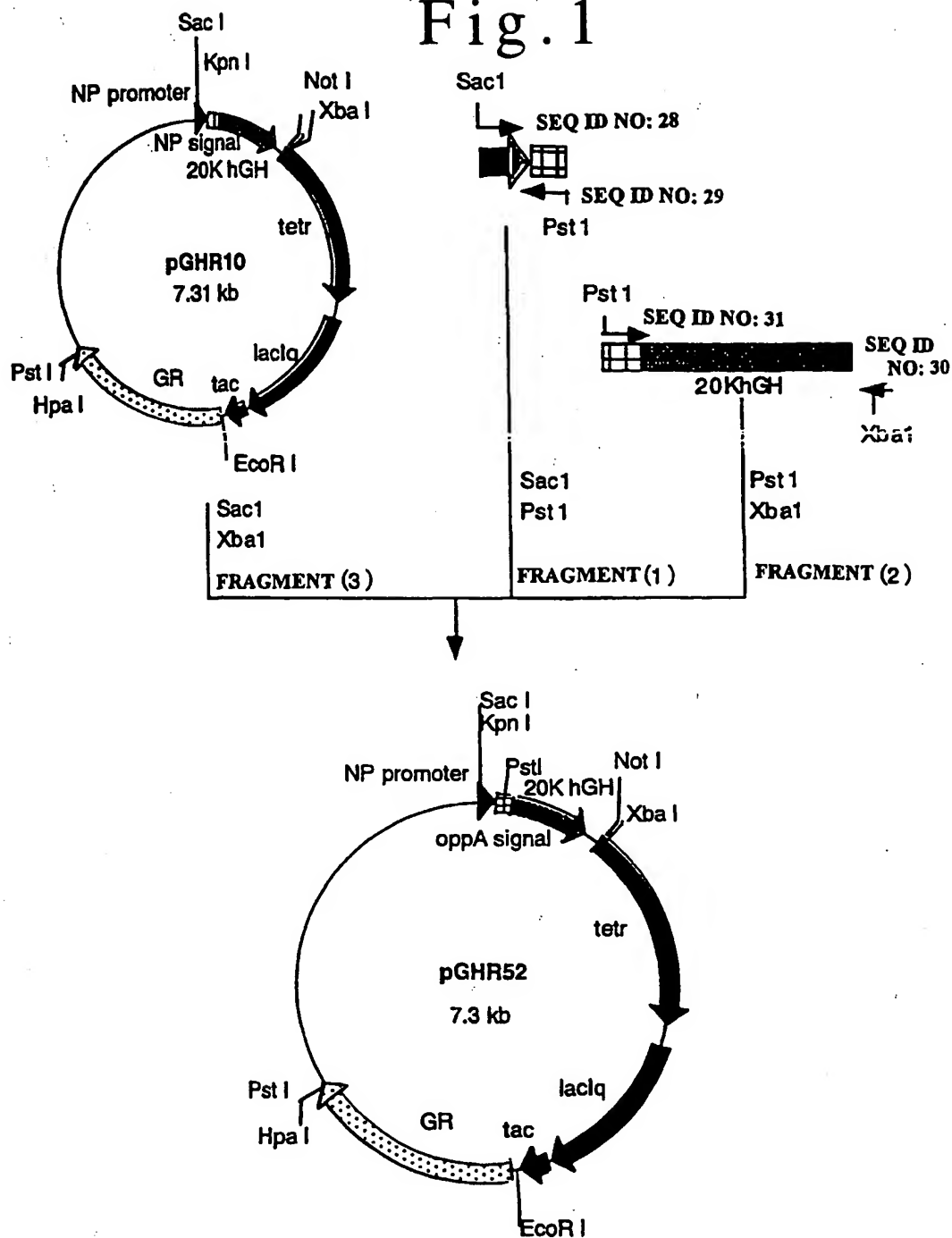
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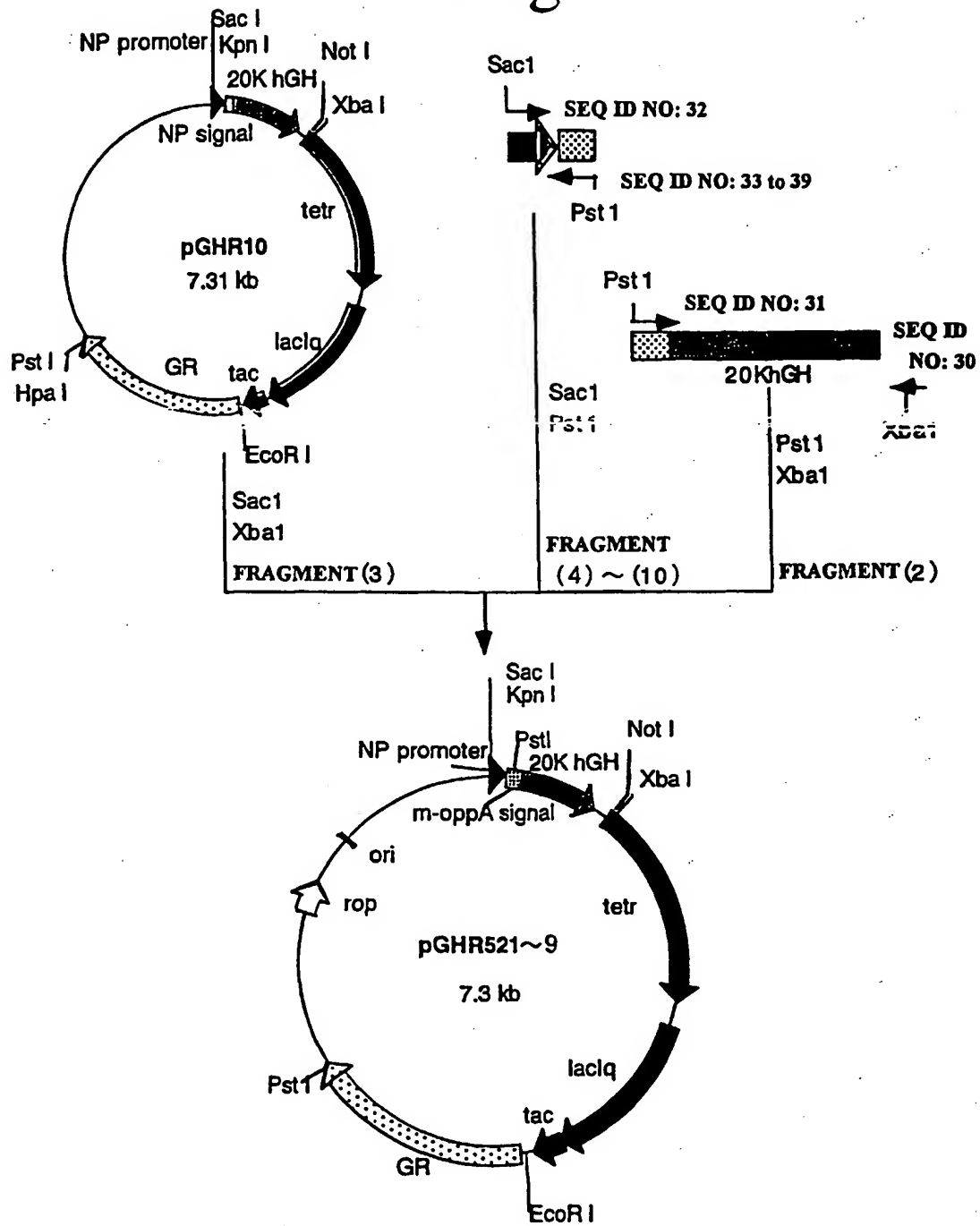
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Fig. 1



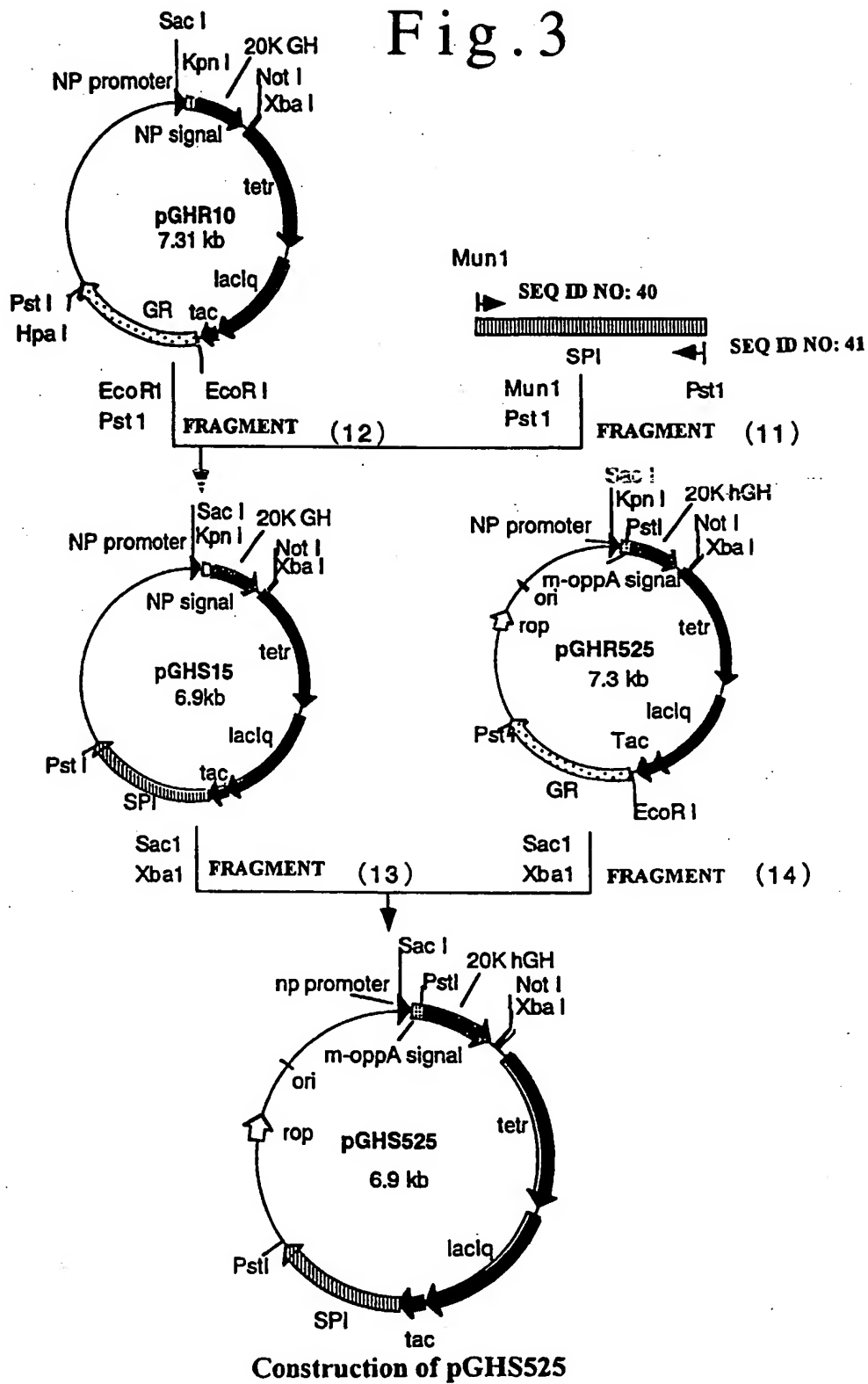
Construction of pGHR52

Fig. 2



Construction of pGHR521 to 529

Fig. 3



(19)



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(12)

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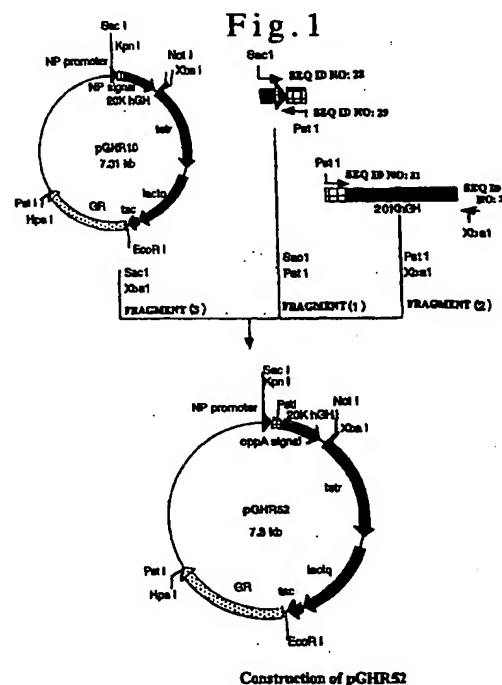
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(54) Method for secretory production of human growth hormone

(57) A DNA encoding 20K hGH is connected directly to a gene encoding Escherichia coli OppA protein secretion signal, or a modified form thereof, and a DNA encoding signal peptidase 1 to construct a recombinant plasmid. E. coli is transformed by said plasmid and cells of the resulting E. coli transformant strain are cultured for secretory production of the 20K hGH in the E. coli periplasm. This method enables efficient secretory production of 20K hGH and easy isolation and purification of 20K hGH from the periplasm fraction because the level of impure proteins in the E. coli periplasm is low.

**EP 0 974 654 A3**



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 99 30 5448

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Y	EP 0 306 673 A (ENIRICERCHE SPA) 15 March 1989 (1989-03-15) * the whole document *	14-19	C12N15/18 C12N15/62 C12N15/31 C12N15/55 C12N1/21 C07K14/61
A	---	1-13	
Y	KASHIWAGI K. ET AL.: "Identification of the polyamine-induced protein as a periplasmic oligopeptide binding protein" J. BIOL. CHEM., vol. 265, no. 15, 25 May 1990 (1990-05-25), pages 8307-8311, XP002129429 * figures 5,6 *	14-19	
A	---	1-13	
A	EP 0 587 427 A (MITSUI TOATSU CHEMICALS) 16 March 1994 (1994-03-16) * the whole document *	1-19	
A	VAN DIJL J ET AL: "Signal peptidase I of Bacillus subtilis: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases" EMBO JOURNAL,GB,OXFORD UNIVERSITY PRESS, SURREY, vol. 11, no. 8, 1 January 1992 (1992-01-01), pages 2819-2828, XP002087013 ISSN: 0261-4189 * the whole document *	14-19	TECHNICAL FIELDS SEARCHED (Int.Cl.7) C12N C07K
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 1 February 2000	Examiner Galli, I
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EUROPEAN SEARCH REPORT

Application Number
EP 99 30 5448

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
A	<p>ZHANG Y -B ET AL: "Analysis of a Streptococcus pneumoniae gene encoding signal peptidase I and overproduction of the enzyme"</p> <p>GENE: AN INTERNATIONAL JOURNAL ON GENES AND GENOMES, GB, ELSEVIER SCIENCE PUBLISHERS, BARKING, vol. 194, no. 2, 31 July 1997 (1997-07-31), pages 249-255, XP004093351</p> <p>ISSN: 0370 1110</p> <p>* the whole document *</p> <p>-----</p>	14-19	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
THE HAGUE		1 February 2000	Galli, I
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01-02-2000

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0306673 A	15-03-1989	IT 1228925 B	10-07-1991
		AT 87654 T	15-04-1993
		DE 3879823 A	06-05-1993
		ES 2053635 T	01-08-1994
		JP 1988640 C	08-11-1995
		JP 2000446 A	05-01-1990
		JP 7004254 B	25-01-1995
		US 5268277 A	07-12-1993
EP 0587427 A	16-03-1994	DE 69318909 D	09-07-1998
		DE 69318909 T	24-12-1998
		JP 6269292 A	27-09-1994
		US 5496713 A	05-03-1996

EPO FORM P4459

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